SCIENTIA MARINA 76(1)
March 2012, 173-176, Barcelona (Spain)
ISSN: 0214-8358
doi: 10.3989/scimar.03399.16B

The use of energy dispersive X-ray spectroscopy to detect strontium marks in fish otoliths

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SUMMARY: Otolith marking provides a reference point for otolith growth patterns by validating the temporal significance of growth increments. This widespread method is primarily implemented using fluorescent dyes. The incorporation of a trace element that appears naturally in otoliths offers an alternative approach. *Diplodus annularis* and *Serranus scriba* otoliths were marked with an intramuscular injection of $SrCl_2$ diluted in 0.9% sterile saline solution (55 mg Sr ml^{-1} saline solution), given at a dose of 100 mg Sr kg^{-1} fish. At 277 to 366 days after marking, the fishes showed little or no mortality and experienced growth in length and weight. All of the otoliths analyzed showed a clear Sr mark detected with backscattered or secondary electron imaging during a scanning electron microsope (SEM) analysis. The mark was confirmed by Sr analysis using an X-ray detector and an energy dispersive spectrometer system with the SEM. The otoliths continued to grow after the mark, and background Sr values in this new growth were much lower than at the mark. This method was feasible and yielded good results. However, the method's cost may limit its applicability to experimental studies.

Keywords: otolith fingerprinting, marking, Sr, mortality.

RESUMEN: LA ESPECTROSCOPÍA DE DISPERSIÓN DE ENERGÍAS DE RAYOS X PARA DETECTAR MARCAS DE ESTRONCIO EN OTOLITOS DE PECES. – El marcado de los otolitos permite establecer una referencia que valida el significado temporal de los incrementos de crecimiento. Este método muy extendido en estudios de validación utiliza preferentemente agentes fluorescentes. Un método alternativo puede ser el marcado con un elemento que aparece de forma natural en la composición de los otolitos. Los otolitos de *Diplodus annularis* y *Serranus scriba* se marcaron mediante una inyección intramuscular de $SrCl_2$ diluída en solución salina estéril al 0.9% (55 mg Sr ml^{-1} solución salina) a una dosis de 100 mg Sr kg^{-1} individuo. Los individuos mostraron crecimiento en peso y talla y muy baja mortalidad después de 277 a 366 días tras el marcado. Todos los otolitos analizados con SEM mostraron una marca nítida, detectada tanto con los electrones retrodisperados como con los electrones secundarios. Mediante microanálisis de rayos X con un sistema EDS acoplado al SEM, dicha marca fue identificada como Sr. Los otolitos continuaron su crecimiento tras el marcado con una baja concentración de Sr comparada con la presente en la marca. La aplicabilidad del método se discute considerándose que ofrece buenos resultados. Sin embargo, el relativo coste del mismo limita su aplicabilidad en estudios experimentales.

Palabras clave: microcomposición otolitos, marcado, Sr, mortalidad.

INTRODUCTION

The marking and tagging of individuals is a widespread practice in population dynamics and age validation studies (Wright *et al.* 2002). Calcified structures are frequently marked with fluorescent dyes that are incorporated into the mineralizing surface. Since the 1960s, several markers have been used that emit a specific coloured fluorescence under ultraviolet light (e.g. tetracyclines, orange xylenol and alizarin). However, some of these markers may have usage restrictions or may demonstrate limited success (Wright *et al.* 2002, Morales-Nin *et al.* 2011 and citations therein).

Strontium (Sr) isotopes can substitute calcium (Ca) in the mineralized portion of the otolith, with Sr concentrations proportional to environmental availability and conditions (Campana 1999). Sr/Ca ratios are widely used to study fish migrations between fresh and salt water (Casselman 1982, Tzeng *et al.* 1997, Phillis *et al.* 2011), and the results of these studies suggest that Sr concentrations are dependent on water salinity (Chang *et al.* 2004). The elemental otolith signatures, specifically of Sr, are used as natural tags to distinguish between wild and reared fish (Gibson *et al.* 2010) or to distinguish riverine origins (Kennedy *et al*. 2000). Sr has been used for the mass marking of fingerlings in enriched Sr water (Elsdon and Gillanders 2003, Munro *et al*. 2008) and for transgenerational marking of viviparous fish larvae by injecting $SrCl_2$ into gestating females (Kuroki *et al.* 2010). Therefore, Sr offers a means for marking calcified tissues using a naturally occurring element.

The aim of this study was to investigate the presence and retention of Sr marks during a relatively long period on otoliths in 2 marine fish species to advance the knowledge of advisable and economic tagging methods.

MATERIALS AND METHODS

Experimental fish

Between March and July 2006, *Diplodus annularis* (Linnaeus, 1758) and *Serranus scriba* (Linnaeus, 1758) were captured by hand line and transported in aerated tanks to the Laboratori d'Investigacions Marines i Aqüicultura (LIMIA). On arrival, individuals were marked with an external plastic T-bar anchor tag (Floytag®), kept under controlled conditions in a grey cylindroconical 1000 L tank with an aerated open circulation system, and fed a diet composed primarily of minced fish (*S. maena*, *B. boops*, *T. trachurus*, *S. pilchardus*, *L. vulgaris*, *M. edulis*, *A. antennatus*). Temperature, salinity and pH were measured daily at midday.

After an approximately 10-month acclimation period, individuals were measured and injected at the dorsal musculature, employing a 24G 1" hypodermic needle and a sterile 1-mL syringe with $SrCl_2$ diluted in 0.9% sterile saline solution (55 mg Sr ml^{-1} saline solution) in April 2007. The injection dose was 100 mg Sr kg^{-1} fish weight. At the end of the treatments, the fishes were euthanized by immersion in a lethal dose of MS-222® anaesthetic in January and April 2008. All fishes were weighed at marking to the nearest g (W M) and measured to the nearest cm of total length (TL M) and at sacrifice (W S, TL S). After death, sagittal otoliths were extracted and kept dry. As an economic point of reference, the cost of strontium for marking all fishes in the present experiment was 13.6 € plus VAT.

Otolith analysis

Left sagitta otoliths were weighed (OW) to the nearest mg and embedded in epoxy resin. The core area was identified in the epoxy block, and a thin transversal section was obtained using a diamond-edged precision saw. Otolith sections containing the core were mounted on glass slides using thermoplastic glue (Crystalbond®). The slides were ground with decreasing grain grinding papers (from 800 to 2400 μm) and polished with cloths and 0.3-μm alumina to obtain otolith sections 200 to 300 μm thick.

The transversal sections were glued with colloidal silver to scanning electron microscope (SEM) stubs, sputter-coated with gold-palladium and observed using a Hitachi S-3500N SEM operated at 5 kV for secondary electron images and 15 kV working with the backscattered electron detector (BSED) and for X-ray microanalysis. For the X-ray microanalysis, a Quantax 400 energy dispersive spectrometer Si(Li) detector (Bruker AXS) with an energy resolution ≤129 eV was used, attached to the SEM. The software used to analyze the spectra and to make the elemental maps was Esprit 1.8 (Bruker AXS).

In each otolith, the Sr band was detected using backscattered electrons (Fig. 1). Different parts of the otolith were selected for analysis to account for possible heterogeneity in the otolith's composition (Payan *et al.* 1999). Once the band mark was detected in a specific otolith area, the procedure was as follows: (1) the distance of the mark band to the edge was measured and the Sr was analyzed in the mark, and (2) the distance of a zone between the band mark and the otolith edge was measured and the Sr was analyzed. The elemental composition was determined in weight percent in a square of 13.48 μm^2 at 4700×. This procedure was repeated 3 times for each otolith, resulting in 6 analyses per otolith.

RESULTS

Tank salinity remained constant at a range between 37 and 37.5 (sd=0.1), whereas pH maintained a range between 8.2 and 8.3 (sd=0.09) during the experimental period.

The weight and total length of fish at marking and sacrifice are summarized in Table 1. The length and weight of both fish species increased during the experimental period, indicating acceptable adaptation to the experimental conditions. *D. annularis* had both a higher growth rate and a lower mortality rate. Notably, no mortalities occurred for *D. annularis* during the experimental period, whereas the mortality rate was 28% for *S. scriba* because of some individual deaths 5 to 8 months after marking, suggesting that the mortality was not directly related to marking.

The fishes were sacrificed at 275 and 366 days after marking. The backscattered electron detector detected Sr in all 18 otoliths (Fig. 1a). The distance between the mark and the otolith edge varied depending on the individual and on the zone of the otolith considered, ranging between 90.84 and 45.26 μm, representing a mean otolith growth of 0.5 μm day^{-1} for *S. scriba*. *D.*

TABLE 1. – Summary of *Serranus scriba* and *Diplodus annularis* total length and weight at marking (TL M cm, W M g), on April 25, 2007 and at sacrifice (TL S cm, W S g). Date of sacrifice and otolith weight (OW, mg) are included. The % weight of Sr in the mark and in the otolith marginal area is also given (sd in brackets) (n=3).

Species	ID	TL M	W M	Date at sacrifice	TL S	W S	OW	Mean %Sr in mark	Mean %Sr after mark
S. scriba									
	3H	18.9	117.9	25/01/2008	19.2	127.5	0.015	3.56 (0.26)	1.80 (0.35)
	8P	16.3	73.2	25/04/2008	17.9	92.1	0.012	1.56 (1.23)	0.81 (0.39)
	12P	20.5	151.5	25/04/2008	22.0	208.7	0.020	3.53 (0.91)	1.32 (0.13)
	14H	20.9	151.1	25/01/2008	22.2	189.9	0.022	3.74 (0.75)	1.19 (0.09)
	22P	16.3	65.1	25/04/2008	18.2	99.6	0.012	3.65 (0.43)	1.05 (0.39)
	23P	15.0	56.2	25/04/2008	18.0	110.7	0.012	2.79 (0.36)	1.18 (0.08)
	24P	16.5	66.4	25/04/2008	18.7	113.5	0.014	2.08 (0.74)	1.23 (0.55)
	34P	18.9	125.7	25/04/2008	20.5	170.9	0.019	3.18 (0.96)	1.23 (0.12)
	43H	-	-	25/01/2008	18.7	109.1	0.013	4.75 (1.07)	1.49 (0.61)
	44H	-	-	25/01/2008	14.2	38.1	0.007	3.00 (1.20)	0.80 (0.36)
mean		17.9	100.9		19.0	126.0	0.015	3.18	1.18
SD		2.2	40.0		2.3	50.8	0.004	1.12	0.41
D. annularis									
	1P	13.8	47.4	25/04/2008	16.8	90.2	0.026	1.49 (0.65)	0.57 (0.24)
	4P	14.1	50.3	25/04/2008	16.4	77.8	0.026	2.92 (0.27)	1.05 (0.39)
	5P	13.4	42.2	25/04/2008	17.3	98.5	0.023	2.08 (0.18)	0.87 (0.35)
	7H	13.5	41.8	25/01/2008	16.1	74.3	0.025	2.23 (0.78)	0.91 (0.26)
	8P	13.4	44.1	25/04/2008	17.2	104.7	0.025	1.56 (0.61)	0.64 (0.14)
	13P	14.7	61.3	25/04/2008	18.3	120.1	0.027	2.05 (0.81)	0.70 (0.01)
	14P	13.6	45.3	25/04/2008	16.4	81.2	0.021	2.44 (0.51)	0.86 (0.48)
	27	-	-	25/04/2008	12.9	34.1	0.013	0.57 (0.08)	0.45 (0.01)
mean		13.8	47.5		16.4	85.1	0.023	1.91	0.76
SD		0.5	6.8		1.6	25.6	0.004	0.82	0.30

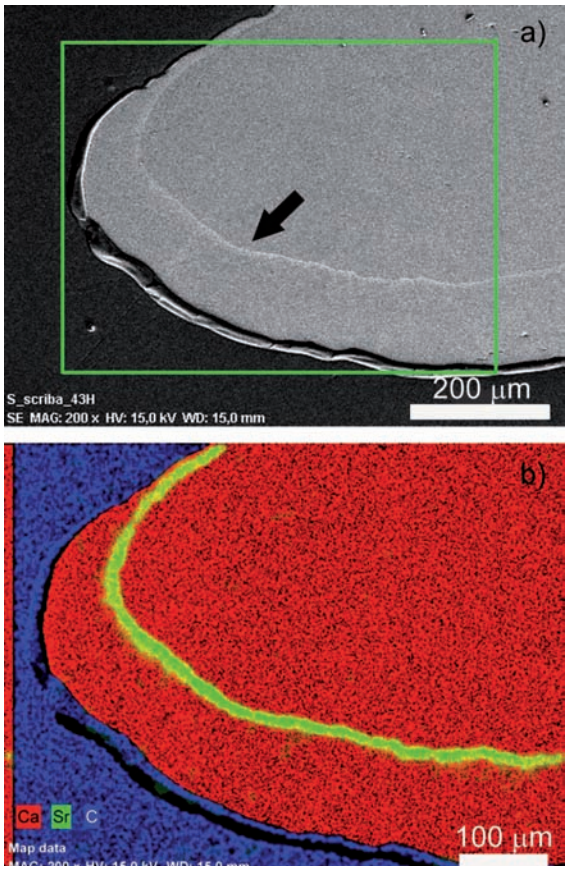

FIG. 1. – Sr band detected in a *Serranus scriba* otolith. (a) Back-scattered electron image at X200 (Sr band, arrow). (b) Elemental mapping of the enclosed area (green square) of (a) showing the two-dimensional distribution of Ca (red), Sr (green) and C (blue).

annularis showed more intense otolith growth after marking, ranging from 164 to 31.78 µm.

Element mapping of the Sr signal provided good results (Fig. 1b), though the procedure was not generally employed because of time constraints. To make a precise map, we acquired the data at 2500 to 3000 counts per second (cps). Thus, 30 min or more were required to obtain an elemental map with fine resolution.

For *S. scriba*, the mean percentage of Sr at the mark was 3.18% (sd=1.12), whereas the mean percentage of Sr was 1.18% (sd=0.41) in the marginal otolith area (Table 1). For *D. annularis,* the mean percentage of Sr at the mark was 1.91% (sd=0.82), whereas it was 0.76% (sd=0.30) in the marginal area (Table 1). A box-plot analysis (Fig. 2) showed clear differences between the 2 species as well as between the mark and marginal otolith areas.

DISCUSSION

For both species, marking individuals with an intramuscular Sr injection led to a null or low mortality rate. All fish of both species showed a clear otolith mark after more than one year of Sr dosage. Because of its detectability and persistence over time, Sr marking appears to be a good tool for obtaining a precise temporal reference mark on the otolith. Though some heterogeneity between otolith zones and individuals was observed (Fig. 2), the mark was always clearly differentiable. Observation with the secondary electron detector of the SEM showed numerous marks and discontinuities along the otolith growth, especially in *D. annularis*, which made the identification of the Sr

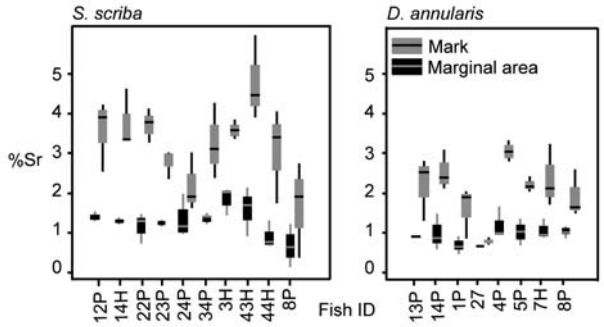

FIG. 2. – Box plots representing the 25%, 50% and 75% percentiles (box) and the non-outlier data range (lines) for mean Sr% in both species and areas. See Table 1 for details on each fish (ID).

mark difficult. Therefore, backscattered electron images (which shows various chemical compositions) and confirmation by Sr analytical detection is recommended as the best option to identify the Sr mark.

There were differences in the amount of Sr fixed in the otoliths between the two species (Table 1 and Fig. 2). The homogeneity of the experimental conditions experienced by both species indicates that otolith incorporation of Sr is species-dependent. Variations of the geochemical signature in the same otolith and between individuals were seen, which is consistent with results found in other species (Tanner *et al*. 2011). This intra-otolith variation is likely due to the lack of spatial uniformity of chemicals within the otolith (Payan *et al.* 1999).

The low mortality rate during the long experimental period shows that the intramuscular injection of $SrCl_2$ at a dosage of 100 mg Sr kg^{-1} fish offered good results, without the medium term teratogenic effects of other markers (Morales-Nin *et al.* 2011). Moreover, Sr appears naturally in otoliths, so there are no limitations on using the marker related to possible effects on the fish or the human consumer. Additionally, this procedure allows for quick marking on board vessels and does not require other burdensome experimental conditions, such as 24-h immersion baths (Elsdon and Gillanders 2003, Munro *et al.* 2008). Furthermore, the marker is both easy to use and available at low cost. On the other hand, SEM-related costs might be a limiting factor. For instance, detecting the Sr mark by BSED imaging and confirming the presence of Sr without quantification may require 10 min per sample. The combination of mark detection using BSED imaging and the validation of Sr presence on several otoliths may provide the best value.

In summary, the benefits of this method (persistence of the mark, low mortality, and ease of marking) must be balanced against its drawbacks (observation time and costs required). The advantages of using this method were demonstrated for experimental studies, particularly when compared with the high mortality and growth disruption experienced by individuals with oxytetracycline marking (Morales-Nin *et al*. 2011).

ACKNOWLEDGEMENTS

This is a contribution of the MICINN-funded project CGL2008-00958. We thank Dr. Ignacio Catalán for his help.

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Scient. ed: S. Somarakis.
Received April 4, 2011, Accepted July 6, 2011.
Published online December 16, 2011.